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(54) Title: ANGIOGENIC POTENTIATING PEPTIDES WHICH POTENTIATE ANGIOGENIC FACTORS

(57) Abstract

Angiogenic potentiating peptides are disclosed which, in some instances, stimulate the growth of endothelial cells, which do not affect the growth of fibroblast cells, and which markedly enhance the angiogenic activity of an angiogenic growth factor, particularly an acidic or basic fibroblast growth factor.

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ANGIOGENIC POTENTIATING PEPTIDES WHICH POTENTIATE ANGIOGENIC FACTORS

FIELD OF THE INVENTION

This invention relates to angiogenic potentiating peptides which potentiate angiogenic factors, and particularly to platelet-derived endothelial cell growth factors which synergize with fibroblast growth factor to enhance angiogensis, plasminogen activator production, cell migration and DNA synthesis in endothelial cells, and capillary proliferation.

BACKGROUND OF THE INVENTION

The term "angiogenic potentiating peptide" refers to a class of relatively small proteins which are capable of potentiating the effects of angiogenic factors. Certain "angiogenic potentiating peptides" may be cell specific growth factors. Two examples of "angiogenic potentiating peptides" are described in greater detail below. These are derived from platelets and cultured vascular smooth muscle cells.

The term "growth factor" denominates a class of relatively small proteins that by themselves or in combination with other proteins increase cellular synthesis of nucleic acids and proteins and increase cellular mass. Conventionally, also, growth factors are considerd to be agents which, by themselves or in combination with other proteins, exhibit mitogenic activity. A variety of growth factors have been isolated and at least partially characterized. See Castor and Cabral, "Growth Factors and Human Disease: The Realities, Pitfalls and Promise," Sem. Arthritis Rheum. 15:33-44, 1985.

Growth factors are also recognized, respectively, to target a variety of cells. Thus, growth factors are known which affect epithelial cells, e.g., the epidermis and connective tissue. Growth factors that affect endothelial cells, which line blood and lymph vessels, are also known. Particularly in connective tissues, where a relatively small number of cells forms a relatively large extracellular matrix, enhanced growth may be manifested not only by cell replicaton but also by increased production of one or more

components of this extracellular matrix. For example, one of the first identified growth factors, the sulfation factor, was determined by its growth-promoting activity as measured by the increased sulfated glycosaminoglycan content of cartilage extracellular matrix.

Although the foregoing recitation suggests that growth factors are primarily concerned with anabolic activities, growth factors are more accurately considered not only to augment cell mass but also certain of their extracellular products which have catabolic impact. One growth factor, for example, connective tissue activating peptide-III, also stimulates the synthesis and secretion of plasminogen activator, a degradative protease discussed in more detail below. Similarly, interleukin-1 also promotes the synthesis and secretion of prostaglandins and collagenase in addition tc its mitogenic effects. Thus, a given growth factor may elicit both anabolic and catabolic behavior from a single target cell.

For definitional purposes, the following nomenclature is adopted in this specification. Growth factor may be abbreviated "GF", epidermal cell growth factor as "EGF", endothelial cell growth factor as "ECGF", fibroblast growth factor as "FGF", basic fibroblast growth factor as bFGF, acidic (or anionic) fibroblast growth factor as aFGF, platelet-derived growth factor as "PDGF", angiogenic factor as "AF", angiogenic potentiating peptide as "APP", platelet-derived angiogenic potentiating peptide as "P-APP", and smooth muscle cell-derived angiogenic potentiating peptide as "SMC-APP." Growth factors are conventionally named after the types of target cells which they are first identified as affecting, i.e., EGF, ECGF or FGF; after the tissue or cells from which they are derived, i.e., PDGF; or after the particular physiological effects which they cause, i.e., GF or AF. Because growth factors are inconsistently named, a given growth factor may confusingly be identified or classifiable by several of the foregoing designations.

The present invention relates to a particular class of APP. An example of an APP which will be discussed in detail is P-APP. A second example is SMC-APP. In addition, the inventors note that certain endothelial cell growth factors (ECGF) derivable from platelets (and thus genercially also a PDGF although PDGF is characterized in the literature as stimulating fibroblasts not endothelial cells), may potentially have activity as angiogenic factors (AF). This class of peptide also surprisingly potentiates the angiogenic activity of at least one fibroblast growth factor (FGF). The basic fibroblast growth factor, defined below, exhibits an endothelial cell growth factor (ECGF) activity and stimulates angiogenesis (and is thus also properly termed an angiogenic factor). The potentiating platelet-derived factor of the present invention is identified herein as "P-APP". The invention will now be described in terms of P-APP. However, this is not intended to limit the scope of the appended claims.

Angiogenic factors have been generally defined as a subset of endothelial cell regulatory factors which may stimulate a group of cellular responses, including (1) an increased rate of endothelial cell proliferation; (2) an increased endothelial cell protease synthesis; (3) chemotactic endothelial cell migration toward a fixed source of the angiogenic factor; and/or (4) capillary proliferation in vivo. It has been observed that substances classified as angiogenic factors may act on DNA synthesis in endothelial cells, thereby increasing the rate of endothelial cell proliferation and thus the rate at which new blood vessels are formed.

Interrelated with the cell proliferation, or mitogenic, AF properties is the general ability of endothelial cell growth factors to increase protease synthesis by endothelial cells. Proteases of particular clinical interest include plasminogen activator and collagenase. Angiogenic factors are able to stimulate synthesis of plasminogen activator and latent collagenase by endothelial

cells, and it is known that plasminogen activator can convert the zymogen plasminogen into plasmin, a protease of wide specificity, which in turn can convert latent collagenase into active collagenase. These two proteases, plasmin and active collagenase, are capable of degrading most of the proteins in surrounding tissues, thus allowing increased invasion of various tissues by cells, such as capillary endothelial cells during neovascularization. Plasminogen activator, particularly tissue plasminogen activator, has great utility in therapy for myocardial infarction by dissolving clots which may occlude coronary arteries. Angiogenic factors may also be chemotactic for endothelial cells including capillary endothelial cells.

With these properties in mind, it had been postulated that the isolation of an appropriate angiogenic factor would lead to the development of therapeutc compounds capable of increasing the blood supply to an organ. For example, in treating certain myocardial infarctions it would be desirable to stimulate the release of plasminogen activator, and increase the revascularization of the blood supply to the heart where interrupted as a result of the infarction; or in treating chronic obstructions and stimulating the growth of new collateral blood vessels. In addition, the use of an angiogenic factor may stimulate healing in decubitus ulcers, surgical incisions and slowly healing wounds, particularly in geriatric and diabetic patients. Moreover, the application of this material to burns may improve the rate and degree of healing. Therefore, a purified placental angiogenic factor (PAF) (subsequently identified as a basic fibroblast growth factor, or bFGF) suitable for therapeutic applications in human beings was sought and discovered (See co-pending application Serial No. 163,142, of Moscatelli et al. for Human Angiogenic Factor Capable of Stimulating Capillary Endothelial Cell Protease Synthesis, DNA Syntehsis or Migration, the disclosure of which is expressly incorporated herein by reference in its entirety). However, as discussed below, the extent to which angiogenesis can be stimulated by presently-known angiogenesis factors appears to be limited.

The bFGF angiogenic factor of the co-pending application has the four above-identified angiogenesis properties, i.e., it is mitogenic, stimulates protease synthesis, is chemotactic and causes in vivo capillary proliferation. This human angiogenic factor was isolated in a substantially purified form from human placental tissues by virtue of its properties of binding strongly to heparin-Sepharose and having a basic pI. The amino acid sequence of this bFGF was also determined. A recombinant-DNA method for the production of this protein was developed. As used in this specification hereinafter, the term bFGF refers to the angiogenic factor of the above-identified copending application and its biological equivalents which stimulate angiogenesis in the same manner although not necessarily to the same degree.

The degree to which angiogenesis can be stimulated by previously known angiogenesis factors appears to be limited: bFGF, for example, can stimulate the growth of bovine capillary endothelial cells only few-fold. It would be useful, therefore, to develop proteins that synergize with bFGF and will thereby potentiate its angiogenic effects. No one thus far has described a protein that synergizes with bFGF to potentiate angiogenesis.

Several endothelial cell specific growth factors have been purified from platelets. Some of these factors also may be capable of acting as angiogenic potentiating peptides, however, this activity never been previously described. Two endothelial cell growth factors have been described by K. Miyazono et al., in "Purification and Properties of an Endothelial Cell Growth Factor From Human Platelets," Journal of Biological Chemistry, 262(9):4098-5103, 1987 and G. King and S. Buchwald, in "Characterization and Partial Purification of an Endothelial Cell Growth Factor from Human Platelets", Journal of Clinical Investigation, 73(2):392-96, 1984.

An additional endothelial cell growth factor has been isolated in neural tissue and was apparently identified by some researchers as ECGF (Schreiber et al., Proc. Nat'l

Acad. Sci. USA, 82:6138-6142, 1985), but by others as "acidic" or "anionic" FGF (Thomas et al., Proc. Nat'l Acad. Sci. USA, 82:6409-6413, 1985, and Klagsbrun et al., Proc. Nat'l Sci. USA, 82:805-809, 1985).

As noted above, none of the foregoing endothelial cell growth factors have previously been reocgnized to possess a potentiating or synergistic effect on angiogenic factors such as bFGF. In view of the relatively limited stimulation caused by growth factors which target endothelial cells, it is therefore an object of the present invention to provide novel compositions of matter which potentiate the therapeutic angiogenic effects of other growth factors such as the basic fibroblast growth factor.

SUMMARY OF THE INVENTION

The present invention relates to APPs capable of potentiating at least one angiogenic factor. In one embodiment, this invention relates to a cell- or platelet-derived factor which is an endothelial growth factor capable of potentiating at least one of the angiogenic activities of an angiogenesis factor. This angiogenesis factor is preferably a fibroblast growth factor possessing at least one activity selected from the group consisting of mitogenic activity, chemotactic activity, the ability to stimulate protease synthesis, the ability to stimulate capillary proliferation in vivo, and combinatinos thereof. More preferably, this fibroblast growth factor is the basic fibroblast growth factor identified in copending application Serial No. 163,142, and its biological equivalents, or acidic FGF.

The APPs of the present invention may be characterized in their isolation from blood platelets or smooth muscle cells, but includes other angiogenesis-potentiating peptides which are biologically equivalent to the factors derived from these sources.

In a medication aspect, for the regulation of angiogenesis-related physiological processes, the present invention relates to a therapeutically-effective dose of an angiogenic growth factor in admixture with an APP capable of

potentiating at least one of the activities of the angiogenic factor, together with suitable pharmaceutical carriers and excipients as may be desirable.

In its therapeutic aspect, the present invention relates to the administration of the medication described above. It is contemplated that the therapeutic and medication aspects of the present broadly encompass the treatment of pathologic, traumatic and surgical processes in which the regulation of the angiogenesis process, generally, may be beneficial.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the DEAE-CL6B column chromatography pattern of 0.25m NaCl eluate of Example 3. The A280 (---- line), growth promoting (·----· line) and P-APP activity in the presence of bFGF (^{x}----^{x} line) are shown. Fraction numbers between 66 and 70 contain the P-APP activity.

Fig. 2 illustrates that both the 45,000 dalton and 65,000 dalton fractions contain the P-APP activity. All assays were carried out in the presence of bFGF (^{x}----^{x} line).

Fig. 3 illustrates the synergistic effect of the P-APP of the present invention on the proliferation of bovine capillary endothelial cells (BCE cells). The ·----· line indicates the effect of various concentrations of bFGF on the proliferation of BCE cells. The ^{o}----^{o} line indicates the ^{3}H-thymidine incorporation in BCE cells when the fixed amount of P-APP of Example 7 was incubated in the presence of various concentratinos of bFGF (0-100 ng/ml).

Fig. 4 presents photomicrographs of bovine endothelial cells cultured on a thick collagen matrix. Fig. 4A shows control cells; Fig. 4B shows the effect of treatment with bFGF. Fig. 4C shows no detectable effect on the morphology of these cells in cultures exposed to a P-APP fraction described herein. Fig.4D shows a marked effect on cultures exposed to P-APP and bFGF simultaneously.

Fig. 5 presents photomicrographs of bovine capillary endothelial cells cultured on thick collagen gels. Fig. 5A shows cells maintained in control medium; Fig. 5B shows the effect of treatment with bFGF. Fig. 5C shows no detectable effect on the morphology of these cells in cultures exposed to conditioned medium from rat aortic smooth muscle cells. Fig. 5D shows a marked effect (much greater than that observed with bFGF alone) on cultures exposed to bFGF in the presence of smooth muscle cell conditioned medium.

DETAILED DESCRIPTION OF THE INVENTION

Practice of the present invention requires the isolation and purification of an APP as may be recovered from a platelet lysate, that is capable of potentiating the angiogenic properties of at least one FGF. In its therapeutic mode, practice of this invention involves the co-administration of this P-APP, specifically defined herein as P-APP, with another angiogenic factor, such as the basic fibroblast growth factor.

It is known that platelets are a rich source of cell growth regulatory proteins, including PDGF, transforming growth factor-beta, epidermal growth factor-like protein, endothelial cell growth fator and hepatocyte growth factor, some of which are cationic and others anionic growth factors. The present invention broadly relates to those cell- or platelet-derived APPs which act in a synergistic fashion to potentiate the angiogenic action of other growth factors, particularly the action of fibroblast growth factors. The present invention also relates to a P-APP which is substantially homologous to, immunologically equivalent to, or, most preferably, biologiclaly equivalent to the native potentiating P-APP isolatable from human platelets. By "biologically equivalent," as used in this specification and claims, it is meant that an angiogenesis potentiating peptide possesses the ability to stimulate the angiogenic properties of FGF in the same manner, although not necessarily to the same degree, as the native P-APP.

Although the following examples describe the isolation of such a P-APP via the experimental protocols therein disclosed, alternative isolation procedures exist as are taught by others who have purified proteins from platelets. The following examples are therefore to be considered as illustrative only, and are not to be considered as limitative, in any manner, of the claims which follow.

EXAMPLE 1: Expression of bFGF from E. coli

Basic FGF is produced according to the method disclosed in co-pending U.S. Patent Application 163,142, specifically incorporated herein by reference.

EXAMPLE 2: Purification of bFGF from E. coli cells

Upon induction with IPTG, the recombinant human bFGF in amounts exceeding 2% of the total cell protein or 8 mg/l/$^{A}600$ unit. When cells were grown at 30°C, the synthesized bFGF accumulates in the soluble cell lysate fraction, but when cells were grown at 37°C, a substantial portion of the recombinant protein was found in the insoluble cell lysate fraction.

The recombinant bFGF could be purified by a single-step affinity chromatology of the soluble E. coli cell lysate fraction on heparin sepharose.

EXAMPLE 3: Isolation of P-APP From Human Platelets

Fifty units of outdated platelets obtained from Belle Bonfils Blood Bank were lysed by repeated freeze-thawing and centrifuged at 20,000 x g for two hours. The supernatant was dialyzed against 20 mM $NaPO_4$, pH 7.4 and applied to a DEAE-A50 column (2.5 x 9 cm). The column was washed extensively until A_{280} reached 0.05, then proteins were eluted with a 20 mM $NaPO_4$ buffer containing 0.25m NaCl at pH 7.4. P-APP was recovered in this 0.25 M NaCl elute. Continued step-wise elution with 0.5 M and 1.0 M NaCl eluants yielded fractions containing growth inhibitory factors.

Example 4: Confirmation of Growth-Potentiating Activity on Bovine Capillary Endothelial Cells

The flow-through fraction and the 0.25 M, 0.5 M and 1.0 M NaCl eluates of Example 1 were dialyzed extensively

against 20 mM $NaPO_4$, pH 7.4, for 16 hours and assayed for their effects on ^3H-thymidine incorporation into bovine capillary endothelial cells. For this assay, confluent cultures of bovine capillary endothelial cells were trypsinized and replated sparsely (2 X 10^3 cells/well) with 500 ul of alpha-MEM medium containing 5% calf serum in gelatin-coated 48-well tissue culture plates (Costar). After 48 hr. of incubation, the medium was replaced with fresh alpha-MEM medium containing 0.5% calf serum and the test samples were added to the wells. 12 hr to 24 hr later, [^3H]- thymidine (0.5uCi/well) was added. After an additional 6 hrs, the cells were fixed with ice-cold 5% (w/v) trichloroacetic acid for 20 min. The resulting precipitates were washed extensively with water and solubilized in 200 ul 0.3M NaOh. Ater mixing at room temperature for 15 min., samples were taken for measuring ^3H-radioactivity. The 0.25 M NaCl eluate showed a linear increase in thymidine incorporation over the range of 0 to 130 ug. The flow-through fraction showed a linear increase over the range of 0 to 65 ug and a slight linear decrease over the range 65 to 130 ug. This decrease may be due to growth-inhibitory activity present in the flow-through fraction at that concentration range. The 0.5 M and 1.0 M NaCl eluate fractions contained an inhibitory activity.

EXAMPLE 5: Further Purification of the P-APP

A portion of the 0.25 M eluate of the DEAE-A50 column of Example 3 was dialyzed extensively for 16 hours against 20 mM $NaPO_4$, pH 7.4, and applied to a column of DEAE-CL6B (2.5 x 20 cm). The column was washed with 500 ml of 20 mM $NaPO_4$, pH 7.4, an eluted with a linear gradient (500 ml) of NaCl from 0 to 500 mM in 20 mM $NaPO_4$, pH 7.4, at a flow rate of 40 ml/hr. Fractions of 4 ml were collected and analyzed for growth-potentiating activity in the presence of bFGF (10 ng/ml) of Example 2 and in the absence of bFGF. The P-APP eluted around 150 mM NaCl wth a peak activity between fraction Nos. 66 and 70. The ---- line in Fig. _ indicates A_{280}, the ·-----· line shows growth promoting activity, and the ^{x}----^{x} line indicates P-APP activity in the presence of

bFGF. This dialysis and column chromatography step also removed many other protein contaminants, as illustrated by the A_{280} line in Fig. 1.

EXAMPLE 6: TSK 4000SW Chromatography of the P-APP

The fraction No. 66 of Example 5, corresponding to the peak of activity on Fig. 1, was concentrated by Centricon-10 to 350 ul and applied to a column of TSK 4000SW. Proteins were eluted with 20 mM $NaPO_4$, pH 7.4, at a flow rate of 30 ml/hr. Fractions of 0.5 ml were collected and analyzed for growth-stimulating activity in the presence of 10 ng/ml bFGF. Two P-APP-active fractions, containing proteins having apparent molecular weights of 45,000 and 65,000 daltons by conventional gel filtration techniques, were identified as synergizing with respect to ^{3}H-thymidine incorporation in the presence of 10 ng/ml bFGF, as illustrated in Fig. 2.

EXAMPLE 7: Further Chromatography of the DEAE-A50 Eluates

The 0.5 M NaCl and 1.0 M NaCl eluates from the DEAE-A50 column of Example 3 were dialyzed against 20 mM $NaPo_4$, pH 7.4, and applied to a second DEAE-A50 column. Bound proteins were eluted with a linear gradient of NaCl from 0 to 1.0 M NaCl in 20 mM $NaPo_4$, pH 7.4, at a flow rate of 60 ml/hr. Fractions of 7 ml were collected and analyzed for synergistic growth-promoting activity in the presence of 10 ng/ml bFGF. Fractions 34 to 50 were analyzed for the induction of plasminogen activator activity in bovine capillary endothelial cells. The plasminogen activator activity was assayed by the method described by Moscatelli et al. in Cell 20:343-351 (1980). The results of this assay indicate that the 100 to 150 mM NaCl eluates contain factor(s) that induce plasminogen activator and potentiate the growth of bovine capillary endothelial cells in the presence of bFGF. These proteins may be the same as the proteins eluted from the DEAE-CL6B column of Example 5.

EXAMPLE 8: Synergistic effect of the P-APP with bFGF

The 100-150 mM NaCl eluate of Example 7 was assayed to determine its mitogenic effect on bovine capillary endothelial cells in the presence of various concentrations of bFGF. The ·-----· line shows the effect of bFGF alone on the proliferation of the cells. The °----° line shows the effect when 40 ul of the NaCl eluate of Example 7 was added along with the indicated amount of the bFGF (0-100 ng/ml). The difference between these lines is surprisingly more than an additive effect of P-APP and bFGF; thus P-APP acts synergistically with bFGF.

EXAMPLE 9: Effect of the P-APP From Human Platelets on the Growth of Bovine Capillary Endothelial Cells

Platelet-derived factors of Example 7 were assayed at various concentrations for incorporation of ^{3}H-thymidine in bovine capillary endothelial cells. Samples were assayed with and without 10 ng/ml bFGF. Without bFGF, the platelet-derived factors alone increased ^{3}H-thymidine incorporation four-fold. The addition of bFGF alone increased ^{3}H-thymidine incorporation two-fold over the control and the combination of P-APP plus basic FGF surprisingly increased ^{3}H-thymidine incorporation 16-fold over the control. Similar results for fraction No. 68 of the DEAE-CL6B eluate of Example 5 are summarized in Table I which follows.

Table I. Effect of P-APP on the proliferation of endothelial cells.

Sample	^{3}H-Thymidine incorporation
	cpm/well +/- SD
0.5% Calf Serum	140 +/- 24
bFGF (10 ng/ml)	600 +/- 41
DEAE-CL6B fraction No. 68	673 +/- 142
bFGF (10 ng/ml) + DEAE-CL6B fraction No. 68	6063 +/- 796

EXAMPLE 10: Effect of the P-APP on the Growth of Bovine Capillary Endothelial Cells in the Presence of Acidic FGF

Fraction No. 67 of the DEAE-CL6B eluate of Example 6 was assayed for incorporation of ^{3}H-thymidine in bovine capillary endothelial cells in the absence or presence of 5 ng/ml acidic FGF. The acidic FGF was purchased from R&D Systems, Inc., Minneapolis, Minnesota (Cat. No. 132-FA).

As shown in Table II, the P-APP synergizes with acidic FGF as well as basic FGF. The addition of either P-APP, acidic FGF or basic FGF alone increased ^{3}H-thymidine incorporation three- to four-fold over the control, and the combination of DEAE-CL6B fraction No. 67 (P-APP) and acidic FGF increased ^{3}H-thymidine incorporation 19-fold over the control.

Table II. Effect of P-APP on the proliferation of bovine endothelial cells

Sample	^{3}H-Thymidine incorporation
	cpm/well +/- SD
0.5% Calf Serum	292 +/- 74
basic FGF (5 ng/ml)	1223 +/- 83
acidic FGF (5 ng/ml)	1043 +/- 114
DEAE-CL6B fraction No. 67	1095 +/- 128
basic FGF (5 ng/ml) + DEAE-CL6B fraction No. 67	5713 +/- 661
acidic FGF (5 ng/ml) + DEAE-CL6B fraction No. 67	5765 +/- 819

EXAMPLE 11: Further Chromatographic Purification of the Eluate of Example 3

An aliquot of the 0.25 M NaCl eluate of the DEAE-A50 column of Example 3 was applied to a column containing BioRad A1.5m or G75. Proteins were eluted with 20 mM $NaPO_4$, pH 7.4, at a flow rate of 50 ml/hr. Fractions of 4-5 ml were collected and analyzed for growth-stimulating activity on endothelial cells and 3T3 cells with 10 ng/ml bFGF.

Growth-promoting activity on 3T3 cells was assayed by the method by Raines and Ross in J. Biol. Chem 277:5154-5160 (1982). From these experiments it is evident that both the 65,000 and 45,000 endothelial cell growth factors act synergistically with BFGF on the growth of BCE cells. The two P-APPs do not stimulate the growth of 3T3 fibroblasts, and do not potentiate the growth promoting effects of bFGF in 3T3 fibroblasts.

EXAMPLE 12: Further Chromatographic Purification of the Factor of Example 11

The fractions between Nos. 74 and 86 of the A1.5m column of Example 11 were pooled and dialyzed against 20 mM sodium acetate, pH 4.8, and applied to an SP-C25 column. Bound proteins were eluted with a linear gradient of NaCl from 0 to 1.0 M NaCl. Fractions of 2 ml were collected and assayed for their effect on growth of bovine capillary endothelial cells and 3T3 fibroblast cells. Results indicate that the endothelial cell growth stimulator proteins have no effect on the growth of 3T3 fibroblasts.

EXAMPLE 13: Further Purification and Characterization of Platelet-derived Factors of Example 6

The proteins, 45,000 dalton and 65,000 dalton, will be further purified separately by using SP-C25 column (20mM Na acetate, pH 4.8) and Mono-Q column (25mM Tris-HCl, pH 7.4). The pure proteins will be desalted on HPLC-C8 column and will be subjected to N-terminal amino acid sequencing analysis. Some of the desalted proteins will be treated with proteolytic enzymes such as trypsin to generate small peptide fragments. Amino acid sequence of these peptide fragments will be determined in order to generate oligonucleotide probes for screening tissue or cell sources of these proteins. The proteins will also be used to prepare antibody against the proteins. The anti-platelet-derived APP will be used to screen tissue or cell sources of these proteins. Once tissue or cells which contain these proteins are identified, cDNA libraries will be prepared and screened for the gene or genes which encodes these proteins.

EXAMPLE 14: In Vitro Angiogenesis Assay

The initial phases of angiogenesis (i.e., activation of quiescent endothelium, cell migration, and invasion of the pericellular matrix) can be mimicked in vitro using capillary endothelial cells cultured on a thick collagen matrix according to the procedures of Montesano et al., Cell 42:469-477, 1985; and Motesano et al., Proc. Nat'l Acad. Sci. USA 83:7297-7301, 1986, the disclosures of which are expressly incorporated herein by reference in their entireties. Bovine adrenal capillary endothelial cells were grown to confluency on 1.0 ml collagen matrices in 12-well (35 mm diameter) tissue culture plates. Cells were maintained in 5% newborn calf serum (NCS) in alpha MEM medium. The collagen matrices were prepared from Types I and III Collagen (obtained from Vitrogen, Collagen Corp.) using the protocols recommended by the manufacturer. Confluent cells were maintained for 48 hrs in 5% NCS/alpha MEM under the following conditions: (i) without further additions as a control; (ii) with the addition of 10 ng/ml bFGF; (iii) with the addition of 80 ul/ml of fraction No. 68 of Example 5; and (iv) with the addition of 10 ng/ml bFGF + 80 ul/ml DEAE-CL6B fraction No. 68.

The results of this assay are presented by Fig. 4 which are photomicrographs of the cell cultures. Cells maintained on the surface of three-dimensional collagen gels formed and remained as a monolayer of closely opposed cells (Fig. 4A). In cultures treated with bFGf (10 ng/ml) alone, numerous endothelial cells could be distinguished by an irregular and dendritic morphology, and a shift in their plane of focus to beneath the monolayer (Fig. 4B). Addition of the protein from fraction No. 68 alone had no detectable effect on the morphology of confluent endothelial cells (Fig. 4C). In cultures exposed to bFGF and the protein from fraction No. 68 simultaneously, the culture morphology was markedly changed (Fig. 4D). In these cultures, more dentritic cells were observed and, additionally, the cells were organized into short branching chords that formed a discontinuous

network under the surface monolayer. In conclusion, the P-APP of DEAE-CL6B fraction No. 68 significantly potentiated the ability of bFGF to induce endothelial cell invasion and tube formation, and thus markedly enhances the angiogenic process.

EXAMPLE 15: Detection of a factor derived from cultured smooth muscle cells with potentiates the angiogenic effects of bFGF on capillary endothelial cells.

Rat aortic smooth muscle cells were grown to overconfluency in 5% NCS/Waymouth's medium in 150-mm diameter tissue culture dishes. The medium was removed and the cultures were washed, then maintained for 30 hours in Waymouth's medium containing 0.1 mg/ml BSA and no serum. This "conditioned medium" was removed, clarified by centrifugation, and stored at -70°C. In vitro angiogenesis assays were performed exactly as described above in Example 14. Confluent endothelial cells cultured on thick collagen gels were maintained for 48 hours in the presence of 5% FBS under the following conditions: (i) in alpha MEM medium without additions as a control; (ii) in alpha MEM medium supplemented with 10 ng/ml bFGF; (iii) in medium conditioned by smooth muscle cells; and (iv) in smooth muscle cell conditioned medium supplemented with 10 ng/ml bFGF.

The results of this assay are presented in Figure 5 which are photomicrographs of the cell cultures. Control cells (Fig. 5A) formed and remained as a monolayer of closely opposed cells on the surface of the collagen plates. In cultures treated with FGF alone (Fig. 5B), numerous endothelial cells which had invaded the underlying collagen could be distinguished by their location (under the monolayered culture) and their irregular and dendritic morphology. Maintenance of the endothelial cells in medium conditioned by smooth muscle cells had no detectable effect on their morphology (Fig. 5C). Addition of 10 ng/ml bFGF to cultures maintained in medium conditoned by smooth muscle cells, however, resulted in marked alterations in the morphology of the cultures. In these cultures (Fig. 5D), many more dendritic cells were

observed under the plane of the monolayer. A factor secreted by cultured vascular smooth muscle cells therefore significantly potentiated the ability of bFGF to induce endothelial cell invasion and tube formation, and thus markedly enhances the angiogenic process.

EXAMPLE 16: Isolation of a factor which potentiates the effects of bFGF from smooth muscle cell conditioned medium

Ten 150-mm cultures of confluent rat vascular smooth muscle cells will be used to generate 100 ml of serum-free conditioned medium. Cultures will be maintained in 5% NCS/Waymouth's medium, washed and then maintained for 30 hours in serum-free Waymouth's medium containing 0.1 ng/ml BSA. The conditioned medium will be clarified by centrifugation, dialyzed aginst 20 mM $NaPO_4$, pH 7.4, then applied to a 2.5 x 9 cm DEAE-A50 column. The column will be washed extensively until the A_{280} of the eluant reaches 0.05. Proteins will be recovered from the flow-through fraction and from fractions eluted by step-wise application of 0.25M, 0.5 M, and 1.0 NaCl washes. Each of these fractions will be tested for SMC-APP activity in ^{3}H-thymidine assays using bovine capillary endothelial cells. The DEAE-A50 fraction containing SMC-APP activity will be dialyzed exhaustively against 20 mM $NaPO_4$, pH 7.4 prior to its application to a 2.5 x 20 cm column of DEAE-CL6B. The column will be washed with 500 ml of 20 mM $NaPO_4$, pH 7.4. Proteins will be eluted with a linear gradient (500 ml) of NaCl from 0 to 500 mM in the same buffer, at a flow rate of 40 ml/hr. Fractions of 4 ml will be collected and analyzed for growth-potentiating activity with the bFGF (10 ng/ml) of Example 2, using ^{3}H-thymidine assays to determine DNA synthesis in BCE cells. The fraction(s) corresponding to the peak of SMC-APP activity will be concentrated by Centricon-10 to 350 ul and applied to a column of TSK 4000SW. Proteins will be eluted with 20 mM $NaPO_4$, pH 7.4, at a flow rate of 30 ml/hr. Fractions of 0.5 ml will be collected and analyzed for growth-potentiating activity with 10 ng/ml bFGF.

Further purification of the SMC-APP activity will be effected by dialysis against 25mM Tris-HCl, pH 7.4, concentration by centricon-10 to a 1.0 ml volume, and separation on a Mono-Q HPLC column. Proteins will be eluted by a linear gradient of 0-0.5M NaCl. Fractions corresponding to the peak of SMC-APP activity, as determining by ^{3}H-thymidine incorporation into BCE cells, will be pooled, concentrated, and applied to an HPLC-C8 column. The peak fractions will then be subjected to N-terminal amino acid sequencing either before or after proteolysis and purification of peptides by HPLC. The deduced amino acide sequence will be used as a basis for the generation of oligonucleotide probes which will then be used to isolate clones from a cDNA library prepared from RNA extracted from human vascular SMC. The amino acid sequence of the SMC-APP will be deduced from nucleotide sequencing of appropriate cDNAs.

From the foregoing detailed description and examples, it will be apparent to those skilled in the art that various modifications and variations could be made in the selection of growth factors and their combinations without departing from the scope or spirit of the invention. It is expressly contemplated that the present invention is equally applicable to the utilization of APPs isolated from human or other mammalian platelets, tissues, or cells, their derivatives and analogues, whether isolated from natural sources or genetically engineered, having a similar potentiating effect on angiogenic factors. It is further expressly contemplated that the angiogenic factors potentiated by the APPs used in the process of the present invention will be broadly drawn from the class of angiogenic factors, whether or not also possessing fibroblast growth factor activity or having substantial homology to the bFGF protein used for illustrative purposes in the foregoing examples. Thus, the present invention is not limited to the foregoing description and examples, but is broadly encompassing of the following claims and their equivalents.

WE CLAIM:

1. An angiogenic potentiating peptide (APP) capable of potentiating at least one of the angiogenic activities of an angiogenic factor.

2. The APP of claim 1, wherein said angiogenic factor is a fibroblast growth factor possessing at least one activity selected from the group consisting of mitogenic activity, chemotactic activity, the ability to stimulate protease synthesis and the ability to stimulate capillary proliferation in vivo.

3. The APP of claim 2, wherein said fibroblast growth factor is bFGF.

4. The APP of claim 2, wherein said fibroblast growth factor is acidic FGF.

5. The APP of claim 1, characterized in its isolation from human blood platelets.

6. The APP of claim 5, further characterized by having a molecular weight of about 45,000 daltons, a stimulatory effect on endothelial cells, the absence of a stimulatory effect on fibroblasts and an inability to bind heparin.

7. The APP of claim 5, further characterized by having a molecular weight of about 65,000 daltons, a stimulatory effect on endothelial cells, the absence of a stimulatory effect on fibroblasts and an inability to bind heparin.

8. A medication for the regulation of angiogenesis-related physiological processes, comprising a therapeutically-effective dose of an angiogenic factor in admixture with an angiogenic potentiating peptide capable of potentiating at least one of the angiogenic activities of said angiogenic factor, together with suitable pharmaceutical carriers and excipients.

9. The medication of claim 8, wherein said angiogenic growth factor is a fibroblast growth factor possessing at least one activity selected from the group consisting of mitogenic activity, chemotactic activity, the

ability to stimulate protease synthesis and the ability to stimulate capillary proliferation in vivo.

10. The medication of claim 9, wherein said fibroblast growth factor is bFGF.

11. The method of claim 9 wherein said fibroblast growth factor is acidic FGF.

12. The method of claim 8 wherein said angiogenic potentiating peptide is SMC-APP.

13. The medication of claim 8 wherein said angiogenc potentiating peptide is platelet-derived APP (P-APP).

14. The medication of claim 13, wherein said P-APP is isoalted from human blood platelets.

15. The medication of claim 13, wherein said P-APP is further characterized by having a molecular weight of about 45,000 daltons, a stimulatory effect on endothelial cells, the absence of a stimulatory effect on fibroblasts and an inability to bind heparin.

16. The medication of claim 13, wherein said P-APP is further characterized by having a molecular weight of about 65,000 daltons, a stimulatory effect on endothelial cells, the absence of a stimulatory effect on fibroblasts and an inability to bind heparin.

17. A P-APP, characterized by having a molecular weight of about 45,000 daltons, a stimulatory effect on endothelial cells, the absence of a stimulatory effect on fibroblasts and an inability to bind heparin.

18. A P-APP, characterized by having a molecular weight of about 65,000 daltons, a stimulatory effect on endothelial cells, the absence of a stimulatory effect on fibroblast and an inability to bind heparin.

19. A method for the regulation of angiogenesis-related physiological processes, comprising the administration of a therapeutically-effective dose of an angiogenic factor in admixture with an angiogenic potentiating peptide capable of potentiating at least one of the angiogenic activities of said angiogenic factor, together with suitable pharmaceutical carriers and excipients.

20. The method of claim 19, wherein said angiogenic factor is a fibroblast growth factor possessing at least one activity selected from the group consisting of mitogenic activity, chemotactic activity, the ability to stimulate protease synthesis and the ability to stimulate capillary proliferation in vivo.

21. The method of claim 20, wherein said fibroblast growth factor is bFGF.

22. The method of claim 20 wherein said fibroblast growth factor is acidic FGF.

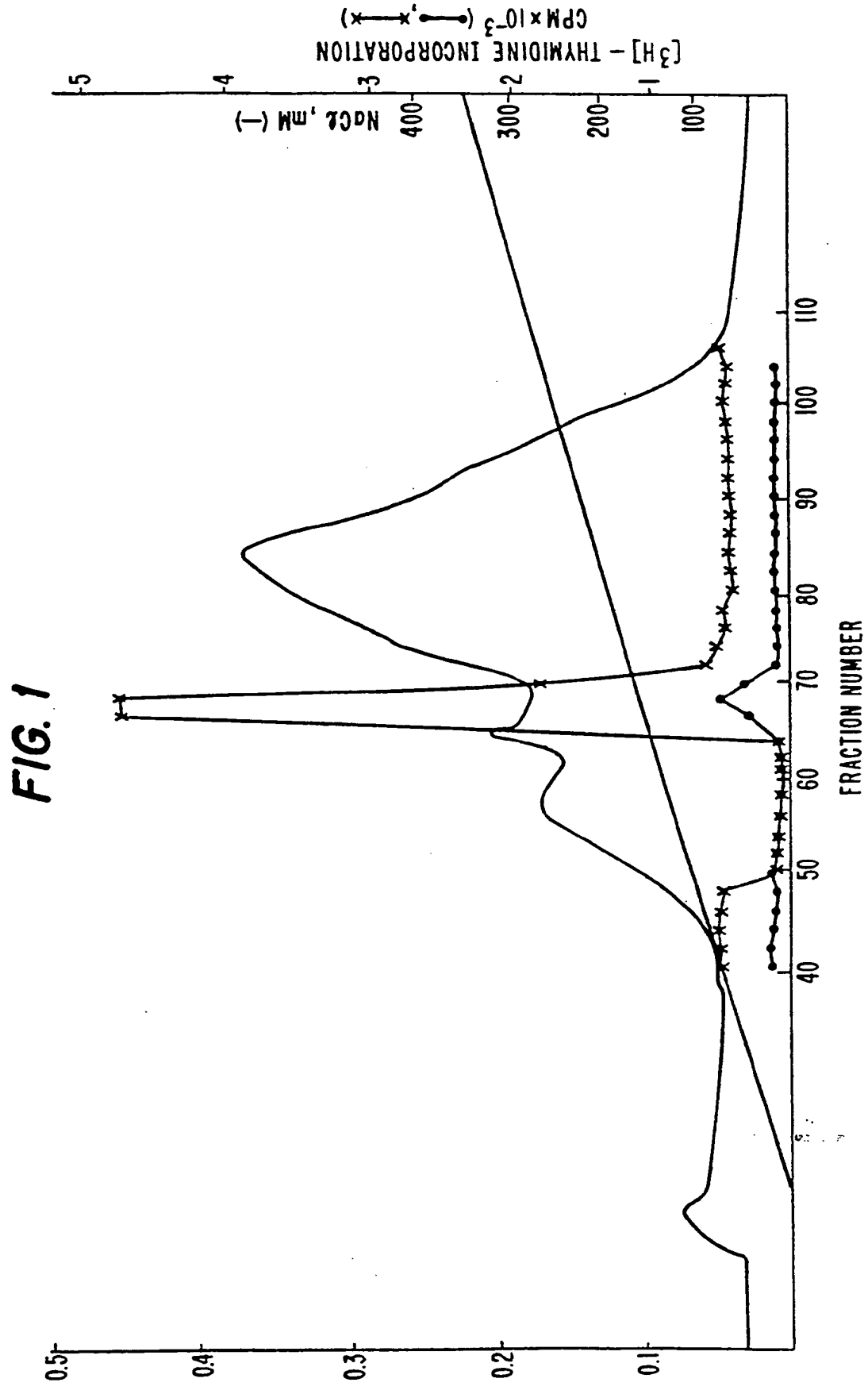
23. The method of claim 19 wherein said angiogenic potentiating peptide is SMC-APP.

24. The method of claim 19 wherein said angiogenic potentiating peptide is P-APP.

25. The method of claim 24, wherein said P-APP is isolated from blood platelets.

26. The method of claim 24, wherein said P-APP is further characterized by having a molecular weight of about 45,000 daltons, a stimulatory effect on endothelial cells, the absence of a stimulatory effect on fibroblasts and an inability to bind heparin.

27. The method of claim 24, wherein said P-APP is further characterized by having a molecular weight of about 65,000 daltons, a stimulatory effect on endothelial cells, the absence of a stimulatory effect on fibroblasts and an inability to bind heparin.


FRACTION NUMBER
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0.4
0.3
0.2
0.1
40
50
60
70
80
90
100
110
NaCl, mM (—)
100
200
300
400
[3H] – THYMIDINE INCORPORATION
CPM × 10^-3 (•—•, ×—×)
1
2
3
4
5

FIG. 1

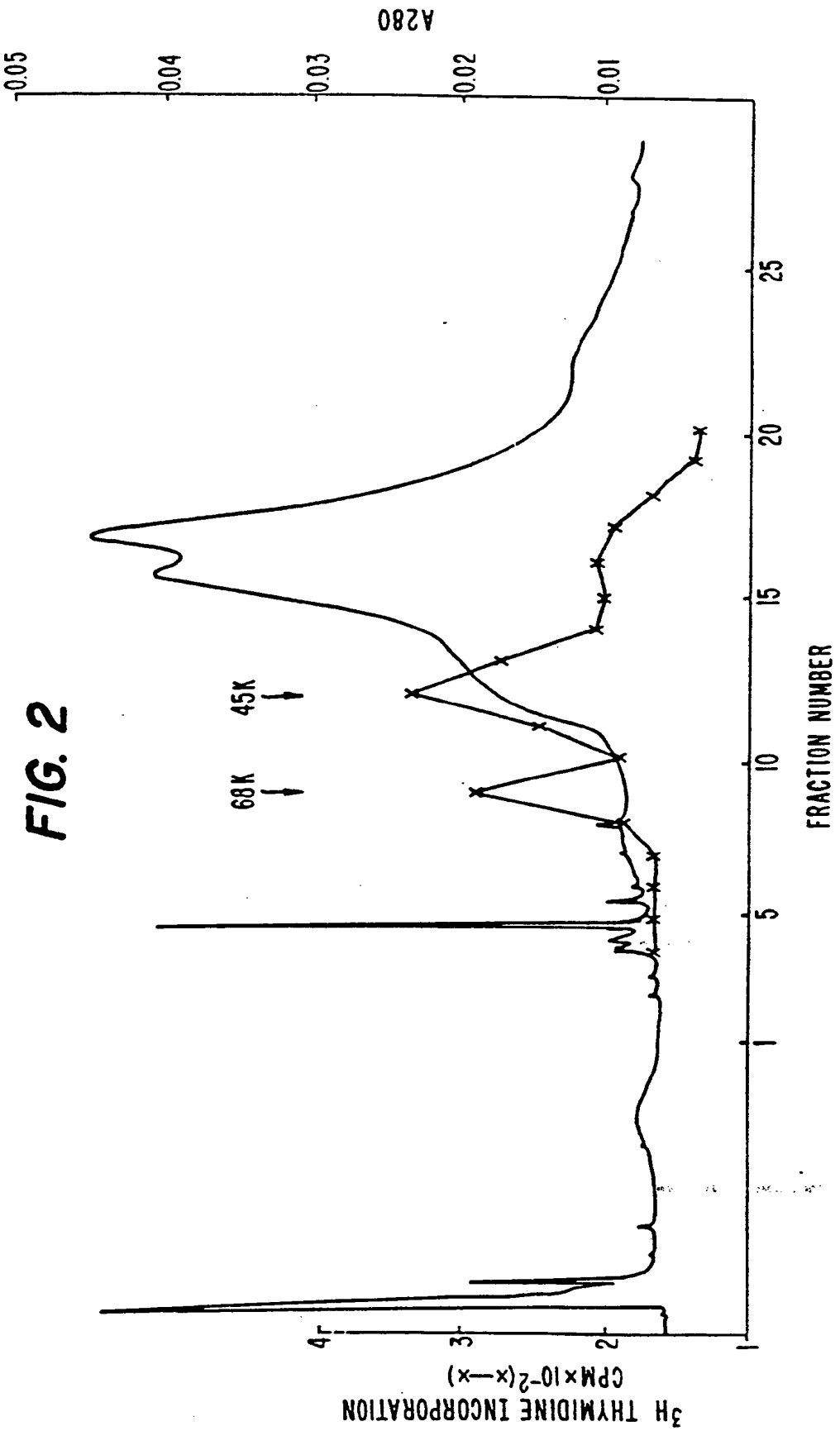

A280
0.05
0.04
0.03
0.02
0.01
45K
68K
FRACTION NUMBER
1
5
10
15
20
25
^{3}H THYMIDINE INCORPORATION
CPM×10^{-2}(x—x)
4
3
2
1

FIG. 2

FIG. 3

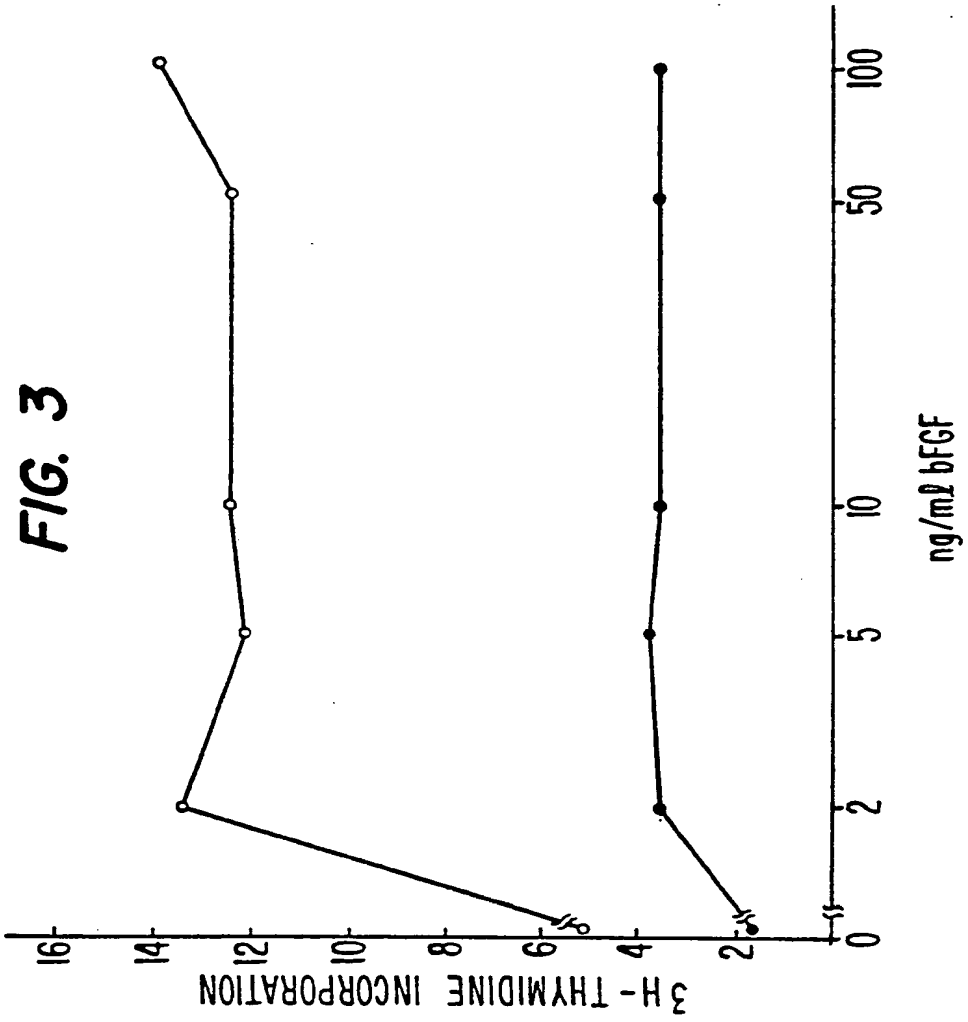

3H-THYMIDINE INCORPORATION
16
14
12
10
8
6
4
2
0
0
2
5
10
50
100
ng/ml bFGF

FIG. 4A

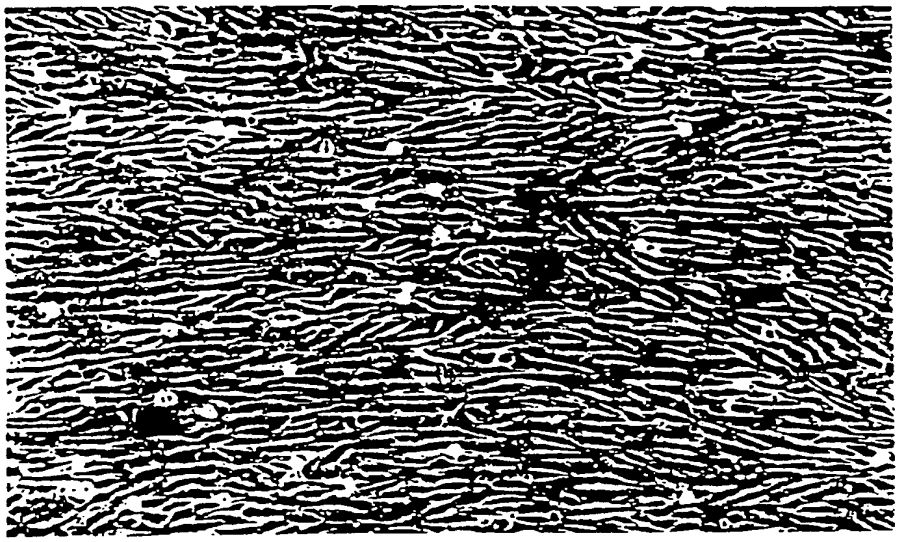


FIG. 4B

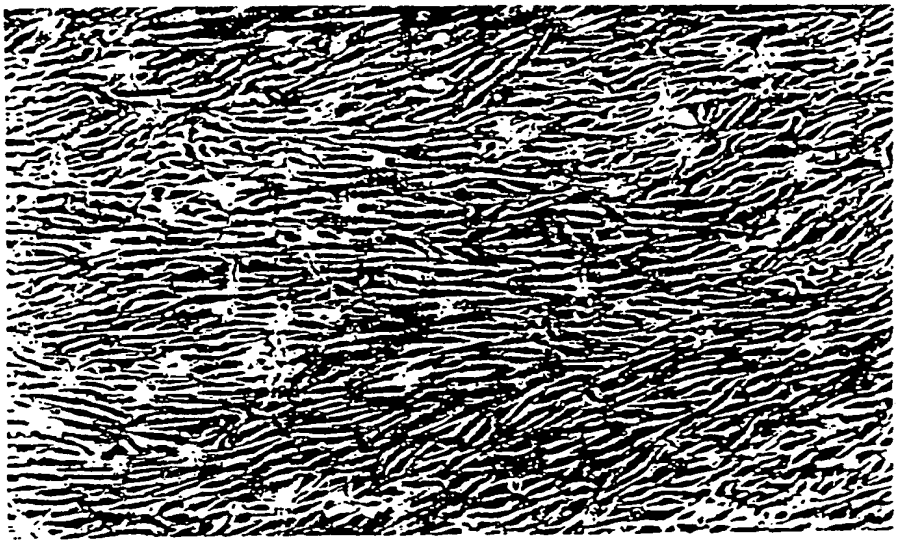


FIG. 4C

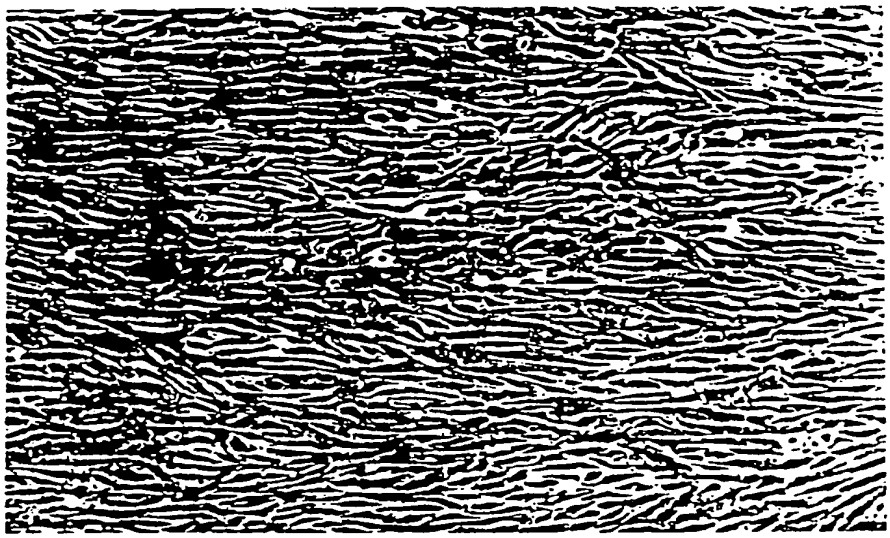


FIG. 4D

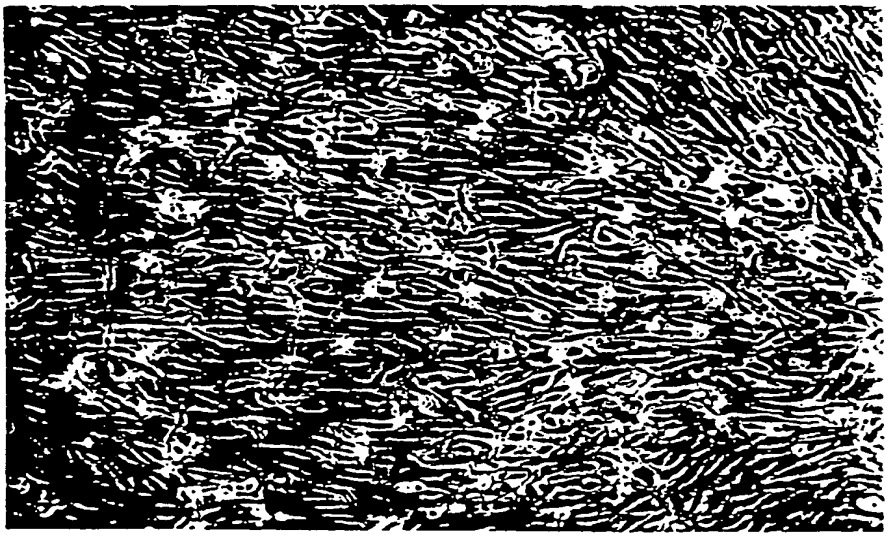


FIG. 5A

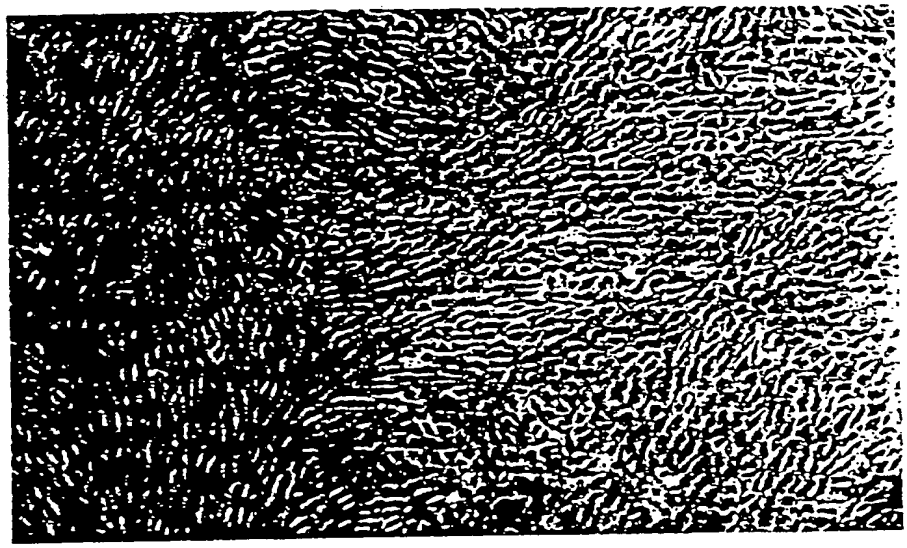


FIG. 5B

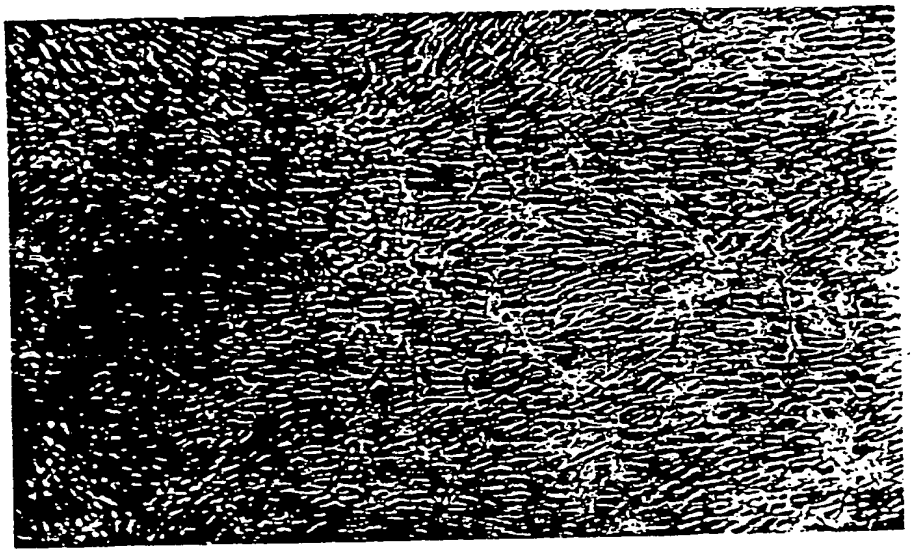


FIG. 5C

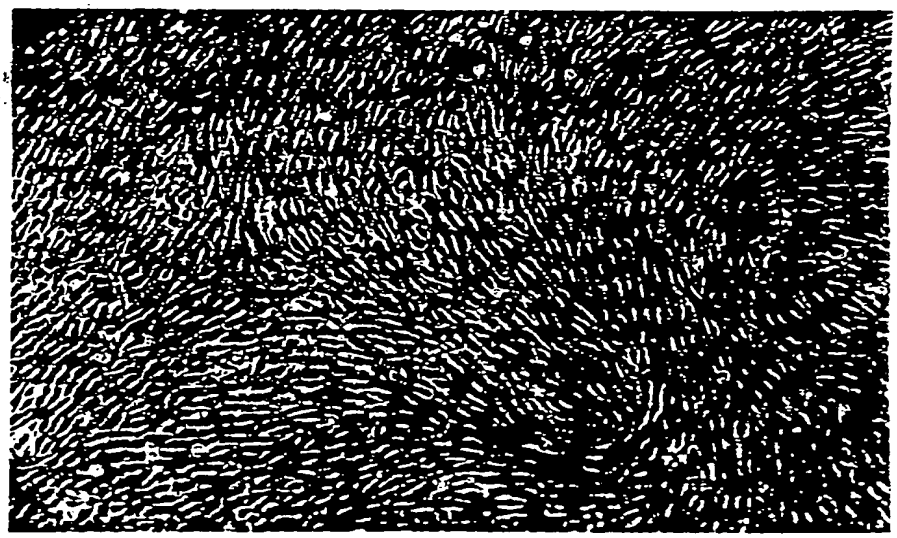
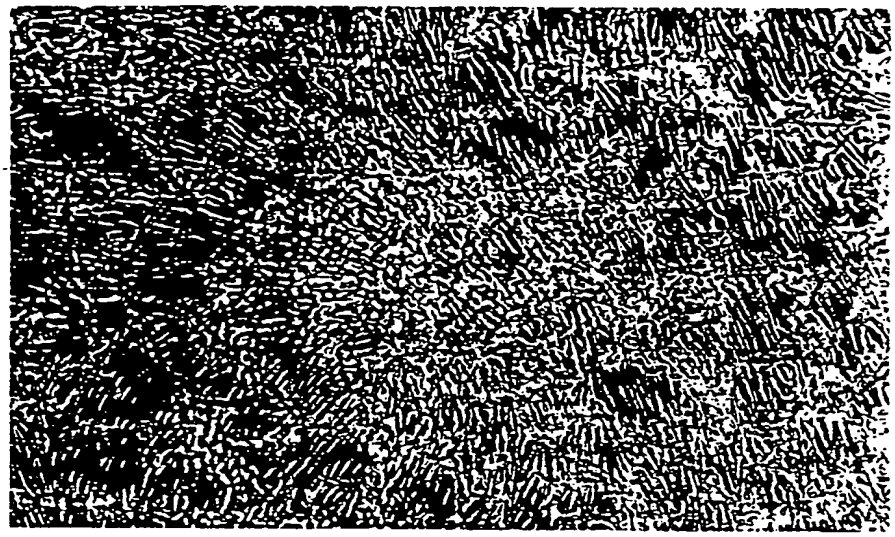


FIG. 5D



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/02699

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4) C07K 7/10; A61K 37/02; A61K 37/36
US Cl. 530/350; 530/399; 530/324; 514/12; 514/21

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US	530/350; 530/399; 530/324; 514/12; 514/21

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

CAS ON LINE

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	US,A 4,444,760 (THOMAS, Jr.) 24 April 1984 (24-04-84) "Purification and Characterization of A Protein Fibroblast Growth Factor" see the abstract.	1-5
Y	US,A 4,529,590 (LEVEEN) 16 July 1985 (16-07-85) "Production of Angiogenic Factor" see the abstract and column 2, Summary of the Invention.	8-14, 19-25
X	US,A 4,727,137 (VALLEE) 23 February 1988 (23-02-88) "Purified Protein Having Angiogenic Activity and Methods of Preparation" see the entire document particularly the abstract.	1-5,8-14 19-25

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

05 September 1989

Date of Mailing of this International Search Report

10 OCT 1989

International Searching Authority

ISA/US

Signature of Authorized Officer

FATEMEH MOEZIE

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication where appropriate, of the relevant passages	Relevant to Claim No
X Y	US,A 4,721,672 (VALLEE) 26 January 1988 "CDNA and Gene for Human Angiogenin (Angiogenesis Factor) and Methods of Expression" see the abstract and the paragraph bridging pages 7 and 8.	1-5 8-14,19-25
X	JUDITH ABRAHAM, J, of Cell Biochem. Supp. Vol. 6,No.11, Part A, page 50, 1987 "Cloning and Characterization of the Genes for the Angiogenic Proteins, Basic and Acidic Filbrablast Growth Factor" see the abstract.	1-5
X	MOSCATELLI, Proc. Natl. Acad. Sci. USA, Vol. 83, 2091, 1986 "purification of a Factor from Human Placenta that Stimulates Capillary Endothelial Cell Protease Production, DNA Synthesis and Migration", see the abstract on page 2091.	1-5
X	BURGOS, Biol. Abstr. 77(3): 1911,1984 "Angiogenic and Growth Factors in Human Amino-Chorion and Placenta", see abstract no. 17462.	1-7